REMARKS

Applicant has amended claims 1-2 and cancelled without prejudice claims 7-8 drawn to non-elected inventions. No new matter has been introduced by the amendment. In particular, support for amendment to claim 1 can be found, e.g., at page 6, lines 19-23; page 7, lines 3-4; page 9, lines 19-24; and page 13, lines 10-12 and 29-30 of the specification. Support for amendment to claim 2 can be found, e.g., at page 7, line 30 through page 8, line 1; page 14, lines 5-6; page 16, lines 5-7 and 28-30; and page 17, lines 13-15 of the specification.

Claims 1-6 are now pending. Reconsideration of the application, as amended, is requested in view of the following remarks.

Claim objections

Claim 2 was objected to as containing grammatical errors. See the Office Action, page 2, part 1. Applicant has deleted the erroneous terms.

Claims 5 and 6 were objected to for improper multiple dependencies. See the Office Action, page 2, part 2. Applicant would like to point out that the improper multiple dependencies were corrected in the Preliminary Amendment filed January 12, 2001, a copy of which is attached hereto as "Exhibit A."

Rejection under 35 U.S.C. § 112, second paragraph

Claims 1-4 were rejected as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention. Specifically, the Examiner asserted that it is unclear whether a heterologous protein fused to any part of a Caulobacter S-layer protein C-terminal secretion signal would necessarily be insoluble. See the Office Action, pages 2-3, part 3. Applicant has amended claim 1 to recite "... a fusion protein, which is insoluble ..." As such, claim 1 is definite. So are claims 2-4, dependent from claim 1.

Rejection under 35 U.S.C. § 112, first paragraph

Claims 1-4 were rejected for lack of enablement. See the Office Action, pages 3-5, part 4. Specifically, the Examiner asserted that:

(1) in view of Smit et al. (U.S. Patent No. 5,976,864), a skilled artisan would not expect that a fusion protein containing any part of a Caulobacter C-terminal secretion signal is

insoluble, but would recognize that at least amino acids 905-1026 of a Caulobacter crescentus S-layer protein are required; and

(2) neither the specification nor the prior art provides any guidance as to which of the amino acids of the S-layer proteins from other species of Caulobacter are required for secretion and for imparting insolubility on a fusion protein.

Claim 1, as amended, reads as follows:

A method of cleaving a fusion protein, which is insoluble in a medium into which a Caulobacter secretes the fusion protein, into a first component which comprises a Caulobacter crescentus S-layer protein fragment incapable of adhesion to a Caulobacter crescentus cell surface but including a secretion signal, and a second component heterologous to the Caulobacter, the fusion protein containing at lease one aspartate-proline dipeptide at a site of cleavage, wherein the method comprises combining the fusion protein with an acid solution of a strength insufficient to solubilize the fusion protein for a time sufficient for cleavage of the fusion protein at said site of cleavage, and wherein the first component remains insoluble in said acid solution after cleavage.

(1) Note that amended claim 1 requires not merely any part of a Caulobacter C-terminal secretion signal but a Caulobacter crescentus S-layer protein fragment incapable of adhesion to a Caulobacter crescentus cell surface and including a secretion signal. Applicant has provided examples of such fragments in the specification. See, e.g., Examples 1-4 on pages 15-18, and page 9, lines 19-24. Also, the art provides adequate guidance as to how to select a fragment of the Caulobacter crescentus S-layer protein that can be used to practice the claimed method. See, e.g., Smit et al., column 5, line 56 through column 8, line 34.

Smit et al. shows that a fusion protein containing amino acids 944-1026 of the Caulobacter crescentus S-layer protein can be secreted but does not precipitate, and a fusion protein containing amino acids 905-1026 can be secreted and precipitates. The Examiner thus concluded that a fusion protein must contain at least amino acids 905-1026 in order to precipitate. Applicant would like to point out that the Examiner's conclusion is beyond what is supported by Smit et al.'s data, as some of the fusion proteins containing amino acids 945-1026, 943-1026, ... 904-1026 may also be able to be secreted and precipitate, which can be readily determined according to the method described in Smit et al.

In this connection, Applicant would like to bring to the Examiner's attention that it is not necessary to test all species covered by claim 1 to show its operativeness. The law does not impose such a formidable burden on inventors seeking patent protection. "Appellants (here, Applicants) are not required to disclose every species encompassed by their claims even in an unpredictable art" (emphasis original). In re Angstadt, 190 USPQ 214, 218 (CCPA 1976). Such a holding is only reasonable, since it is very difficult, if not impossible, to test and disclose all operative species in the chemical and biotechnology fields. Indeed, as pointed out by the Angstadt court "[w]ithout undue experimentation or effort or expense the combinations which do not work will readily be discovered and, of course, nobody will use them and the claims do no cover them." Id, at 219.

As mentioned above, given the guidance provided in the specification and in the art, a skilled artisan would be able to determine which fragments of the Caulobacter crescentus S-layer protein can be used for practicing the claimed method. No undue experimentation is needed.

(2) Amended claim 1 has been limited to the S-layer protein of Caulobacter crescentus.

For the amendment and reasons set forth above, Applicant submits that claim 1 is enabled. By the same token, claims 2-4 dependent from claim 1 are also enabled.

Rejection under 35 U.S.C. § 103(a)

Claims 1-4 were rejected as being unpatentable over Smit et al. in view of Ausubel et al. (Current Protocols in Molecular Biology, John Wiley and Sons, Inc., 1994) and Better (U.S. Patent No. 5,851,802). See the Office Action, pages 5-7, part 5.

As mentioned above, amended claim 1 is drawn to a method of cleaving an insoluble fusion protein containing a fragment of the Caulobacter crescentus S-layer protein and a component heterologous to Caulobacter. The method involves treating the fusion protein with an acid solution such that an Asp-Pro bond in the fusion protein is cleaved and the fragment of the Caulobacter crescentus S-layer protein remains insoluble after cleavage.

Smit et al., discloses expression and secretion of heterologous polypeptides from Caulobacter. On the other hand, Ausubel et al. discloses hydrolysis of an Asp-Pro bond in a fusion protein at low pH; and Better discloses cleavage of an Asp-Pro bound in an insoluble

fusion protein (Bone D-BPI peptide) at acidic pH, resulting in insoluble Bone D and soluble BPI peptide.

It is the Examiner's position that it would have been obvious to a skilled artisan to combine the teachings of Smit et al., Ausubel et al., and Better to come up with the claimed method, and that one would have been motivated, with a reasonable expectation of success, to do so.

Applicants would like to point out that the references cited by the Examiner, alone or combined, fail to disclose that the fragment of the Caulobacter crescentus S-layer protein would remain insoluble after cleavage at low pH, as required in claim 1. Indeed, it is completely unexpected that the fragment of the Caulobacter crescentus S-layer protein would remain insoluble after cleavage at low pH, given the knowledge in the art that the entire Caulobacter crescentus S-layer protein is soluble at low pH. See, e.g., page 6351, right column, lines 6-12 of Nomellini et al., J. Bacteriol. 179:6349-6354, 1997, a copy of which is attached hereto as "Exhibit B." Note that "presence of an unexpected property is evidence of nonobviousness" (MPEP 716.02(a)).

For the amendment and reasons set forth above, Applicant submits that claim 1, as well as claims 2-4 dependent from it, is patentably distinguishable over the prior art references cited by the Examiner.

CONCLUSION

Applicant submits that the grounds for objection and rejection asserted by the Examiner have been overcome, and the claims, as pending, define subject matter that is enabled and nonobvious. On this basis, it is submitted that allowance of this application is proper, and early favorable action is solicited.

Exhibit-A

YRT

F+R-3

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The Patent and Trademark Office date stamp sets forth the receipt date (or both the receipt date and the Serial Number) of a patent application identified as follows:

Applicant: John Smit

Title: CLEAVAGE OF CAULOBACTER PRODUCED RECOMBINANT FUSION PROTEINS

[X] Transmittal Letter
[X] Appln 26 Pages of Spec. 2 Pages of Claims 8 Total Claims 1 Pages of Abstract
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( ) **Total number of claims in the application, noted in the transmittal letter and on the filing receipt match**

( ) **Number of Independent claims in the application and noted in the transmittal letter and on the postcard match**

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( ) **Inventor(s) named on the filing receipt and the inventor(s) named in the declaration/filing request match**

( ) **Title noted on filing receipt and on the application match**

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Attorney's Docket No.: 08106-004001 / 82104-17

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : John Smit
Serial No. :
Filed : Herewith
Title : CLEAVAGE OF CAULOBACTER PRODUCED RECOMBINANT FUSION PROTEINS

Art Unit : Unknown
Examiner : Unknown

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Commissioner for Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Prior to examination, please amend the application as follows:

In the Claims:

In claim 3, line 15, delete "or 2".

In claim 4, line 18, delete "or 2".

In claim 5, line 21, please delete "any one of claims 1-4" and insert therefore --claim 1--.

In claim 6, line 24, please delete "any one of claims 1-5" and insert therefore --claim 1--.

In claim 7, lines 27-28, please delete "suitable for use in the method of claim 1, wherein the method".

In claim 8, line 8, please delete "as described in" and insert therefore --by the method of--.

CERTIFICATE OF MAILING BY EXPRESS MAIL

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I hereby certify under 37 CFR §1.10 that this correspondence is being deposited with the United States Postal Service as Express Mail Post Office to Addressee with sufficient postage on the date indicated below and is addressed to the Commissioner of Patents, Washington, D.C. 20231.

January 12, 2001
Date of Deposit

Samantha Bell
Signature

Samantha Bell
Typed or Printed Name of Person Signing Certificate

REMARKS

All amendments are to remove multiple dependencies or to clarify the claims language. No new matter has been added.

Applicant submits that all of the claims are now in condition for examination, which action is requested. Please apply any charges or credits to Deposit Account No. 06-1050.

Respectfully submitted,

Date: 1-12-01

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Exhibit-B

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Factors Controlling In Vitro Recrystallization of the *Caulobacter crescentus* Paracrystalline S-Layer

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The S-layer of *Caulobacter* is a two-dimensional paracrystalline array on the cell surface composed of a single protein, RsaA. We have established conditions for preparation of stable, soluble protein and then efficient in vitro recrystallization of the purified protein. Efficient recrystallization and long range order could not be obtained with pure protein only, though it was apparent that calcium was required for crystallization. Recrystallization was obtained when lipid vesicles were provided, but only when the vesicles contained the specific species of *Caulobacter* smooth lipopolysaccharide (SLPS) that previous studies implicated as a requirement for attaching the S-layer to the cell surface. The specific type of phospholipids did not appear critical; phospholipids rather different from those present in *Caulobacter* membranes or archaebacterial tetraether lipids worked equally well. The source of LPS was critical; rough and smooth variants of *Salmonella typhimurium* LPS as well as the rough form of *Caulobacter* LPS were ineffective. The requirement for calcium ions for recrystallization was further evaluated; strontium ions could substitute for calcium, and to a lesser extent, cobalt, barium, manganese and magnesium ions also stimulated crystallization. On the other hand, nickel and cadmium provided only weak crystallization stimulation, and zinc, copper, iron, aluminum ions, and the monovalent potassium, sodium, and lithium ions were ineffective. The recrystallization could also be reproduced with Langmuir-Blodgett lipid monolayers at an air-water interface. As with the vesicle experiments, this was only successful when SLPS was incorporated into the lipid mix. The best method for RsaA preparation, leading to apparently monomeric protein that was stable for many months, was an extraction with a low pH aqueous solution. We also achieved recrystallization, albeit at lower efficiency, using RsaA protein solubilized by 8 M urea, a method which allows retrieval of protein from inclusions, when expressed as heterologous protein in *Escherichia coli* or when retrieved as shed, precipitated protein from certain mutant caulobacters. In summary, the clarification of recrystallization methods has confirmed the requirement of SLPS as a surface attachment component and suggests that its presence in a membrane-like structure greatly stimulates the extent and quality of S-layer formation. The in vitro approach allowed the demonstration that specific ions are capable of participating in crystallization and now provides an assay for the crystallization potential of modified S-layer proteins, whether they were produced in or can be secreted by caulobacters.

Caulobacter crescentus and strains related to this species produce S-layers of similar appearance and with comparable structural and biochemical properties (3, 14, 27, 30). The structure is a two-dimensional highly ordered array that is attached to the outer membrane, has no portion that penetrates the outer membrane, and completely covers the bacterium. It is composed of a single protein species with a size of 98 kDa (11) which self assembles into a hexagonally arranged lattice; the prominent morphological unit contains six monomers and is spaced at 22-nm intervals (28). We study the S-layer gene (*rsaA*) and protein (RsaA) of *C. crescentus* CB15 in an effort to define key features of this structure, that is, the factors that control secretion, surface attachment, and self-assembly of the protein into a two-dimensional crystalline structure. The process has been largely empirical; the protein has no signal leader peptide, has neither N- nor C-terminal posttranslational processing, and is not homologous to other secreted proteins (11). The folded structure cannot be predicted with current secondary structure analysis algorithms, the points of attachment to the cell surface are not yet known, and a putative calcium binding motif (11) can only be estimated as a factor in attachment or crystallization.

Given this situation, a reliable method for in vitro crystallization is desirable to assist with the sorting out of structure-function issues. We had previously noted that RsaA, prepared by a low pH or EDTA extraction method, was capable of reforming S-layer, if adequate but not excessive amounts of calcium ions were supplied (30). But the quality of crystallization was far poorer than that reflected in the images that are routinely available from in vivo crystallization. It did, however, demonstrate that RsaA by itself was sufficient for crystallization. Moreover, it is, in principle, possible to get long-range good order from RsaA only. When colonies derived from mutants that fail to attach the S-layer (due to the loss of a specific species of lipopolysaccharide [LPS] termed smooth LPS [SLPS] [31]) are examined, extensive sheets of S-layer which form as a mirror-image double layer of S-layer and show a high degree of order are found (28, 31).

In vitro recrystallization has been accomplished with a variety of other bacterial S-layer systems (15, 23–25); often S-layer proteins derived from gram-positive bacteria will reassemble after simple removal of the dissociating agent (usually a chaotrope), with no requirements for ions or a particular surface. In

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some cases, S-layer proteins will recrystallize on a surface support, but there is a full range of requirements: some will attach only to cell wall layers from which they were isolated, while others will also attach to another cell wall type and still others will even form on nonbiological surfaces (21). Others, particularly S-layers of gram-negative bacteria, require ions, often calcium, to accomplish or at least stimulate in vivo or in vitro crystallization (2, 5, 7, 10, 33). *Spirillum serpens* (now *Aquaspirillum serpens*) apparently requires an LPS-lipid surface (2, 6), but it was found that LPS-lipid complexes from *Pseudomonas aeruginosa* would also serve as a template to stimulate reassembly, indicating a certain generality in the surface requirement.

The requirement for divalent ions for reassembly is also a common theme for gram-negative bacterial S-layers, and we had indications that calcium was a key ion involved with the *Caulobacter* S-layer assembly (28). Recently, we discovered that calcium must also have other roles in the biogenesis of the S-layer; strains selected as capable of growing in the absence of calcium did not produce RsaA in calcium-limited conditions (30). This finding severely limited our ability to assess in vivo the ability of other ions to substitute for calcium in the crystallization process. This limitation and the desire to more directly implicate the role of SLPS in the crystallization/attachment process led us to explore more fully in vitro methods for *Caulobacter* S-layer recrystallization.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *C. crescentus* NA1000 (31) and CB2A (26), a spontaneous S-layer-negative mutant of CB2, were used for most experiments. For some experiments JS1001, a spontaneous mutant of NA1000 which fails to produce SLPS and therefore sheds the S-layer protein, was used (8, 31). Cells were grown in a peptone yeast extract medium supplemented with calcium chloride and magnesium chloride (PYE) (16) at 30°C with shaking. For some experiments, to obtain higher cell yield per liter, the medium was further supplemented with glucose to 0.1% and ammonium chloride to 0.05%.

S-layer protein (RsaA). For wild-type protein and any variants that were still crystallized and surface attached, the method for protein purification was a modification of the method described by Walker et al. (30). Cells were grown in PYE medium to mid- to late-logarithmic phase (optical density at 600 nm [OD_{600}] of 1 to 1.5) at 30°C and were harvested by centrifugation (10,000 to 15,000 × *g* for 15 min). All subsequent manipulations were performed at room temperature unless otherwise specified. Cells were suspended in 50% of the original culture volume in 10 mM HEPES buffer (pH 7.2). After centrifugation, the cells were suspended in 5% of the original culture volume in 50 mM HEPES (pH 2.0). After 5 to 10 min of exposure to this low-pH condition, the suspension was neutralized to pH 7 to 7.5 by addition of appropriate amounts of 10 N NaOH. The cells were then pelleted by centrifugation, and the supernatant was collected. Such preparations contained 1 to 2 mg of RsaA per ml, which remained as a soluble protein solution for several months. For long-term storage at 4°C, sodium azide was added to 3 mM. Prior to use, the preparations were recentrifuged at 15,000 × *g* for 2 min. This procedure was minimally suitable for cell cultures ranging from 10 ml to 1 liter in size.

When it was desirable to test crystallization capability for proteins that were not crystallized and attached to the *C. crescentus* cell surface, that is, they were produced from a "shedding" strain of *Caulobacter* (8, 31), an alternate method was used. In such cases, RsaA was a precipitated protein; in the case of the shedding strains, the protein apparently makes an aberrant attempt to crystallize. Such protein preparations were treated with 8 M urea at room temperature, which was able to solubilize much of the RsaA present. Following centrifugation at 15,000 × *g* to remove residual insoluble protein, the preparations were adjusted to 1 to 2 mg of RsaA protein per ml and dialyzed against deionized water to remove the urea. Preparations containing up to 2 mg of RsaA per ml were stable as soluble solutions; higher concentrations led to precipitation.

Vesicle preparation. Lipid vesicles were prepared by placing 100 μl (2.1 mg) of a lipid mix (see below) in a 1.5-ml microcentrifuge tube and removing the organic solvent with a Speedvac concentrator. When LPS (typically the *Caulobacter* SLPS) was to be included in the vesicles produced, it was added as an aqueous solution at no more than 10% of the organic solvent volume. In the case of SLPS, most experiments were performed with 100 μg of SLPS in 10 μl of water; this is equivalent to a 1:200 molar ratio of SLPS to phosphotidylcholine. Subsequent manipulations were performed at room temperature unless otherwise noted. After the lipid mix was dried, 0.4 ml of 160 mM KCl was added to the tube, and the mix was agitated with a vortexer several times. The mixture was given a sonication treatment, by using a microprobe at the lowest power setting; two or three 10-s bursts with cooling on ice between bursts was sufficient to produce vesicles with diameters in the range of approximately 0.2 to 0.5 μm, with some ranging as high as 2 μm. Water (0.6 ml) was added to the mixture, which was then centrifuged for 3 to 5 min at 15,000 × *g*. The pellet was suspended in 1 ml of deionized water and recentrifuged. The pellet was then resuspended in 0.5 ml of water and stored at 4°C.

For most experiments a lipid mixture consisting of 22.5 mg of dipalmitoylphosphatidylcholine, 9 mg of cholesterol, and 0.75 mg of hexadecylamine dissolved in 1.5 ml of absolute ethanol was used (13). This mixture was stored at −20°C and heated at 65°C briefly to dissolve the lipids before use. For some experiments, a mixture of lipids extracted from the archaebacterium *Methanospirillum hungatei* was used (29). These were suspended to 21 mg/liter in chloroform instead of ethanol. When SLPS was incorporated into these lipids, 50 μl of methanol was also added to the lipids, to allow the subsequent addition of the aqueous SLPS solution without a phase separation. Subsequent steps were the same as described above.

Caulobacter SLPS was prepared as previously described (20, 31). In brief, crude lipids were extracted from cells by an NaCl-EDTA treatment, followed by digestion with proteinase K (to remove residual protein) and preparative sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis to separate the rough LPS and SLPS and any contaminants. The SLPS, which runs as a single band, was excised, electroeluted, concentrated, lyophilized (to determine the weight), rehydrated with deionized water, and stored at 4°C as an aqueous solution at about 10 mg/ml. The rough LPS of *Caulobacter* was excised and processed in the same fashion.

In vitro crystallization of RsaA onto vesicles. In a typical experiment, the incubation mixture contained 190 μl of water, 25 μl of an ion source (typically 5 mM $CaCl_2$), 15 μl of vesicles prepared as described above, and 20 μl of RsaA protein (1 to 1.5 mg/ml). This mixture was incubated at room temperature with continuous inversion for 2 h. The mixture was centrifuged for 5 min, and all but 10 to 20 μl of liquid was removed from the pellet. The pellet was then suspended, portions were mixed with an equal quantity of 2% ammonium molybdate (pH 7.5), and bacitracin was added to 100 μg/ml (final concentration) to facilitate uniform spreading of stain on the grids (12). This mixture was applied to Formvar or Pioloform-filmed 400-mesh nickel grids stabilized with carbon. Samples were examined by electron microscopy at 60 kV.

The suitability of ions other than calcium, as well as higher concentrations of several of the ions, was also evaluated. In place of a 5 mM $CaCl_2$ solution, 5 mM solutions of $SrCl_2$, $MnCl_2$, $MgCl_2$, $NiCl_2$, $AlCl_3$, $CuCl_2$, $CdCl_2$, $ZnCl_2$, $FeCl_3$, $CoCl_2$, $BaCl_2$, NaCl, KCl, and LiCl were substituted. In another set of experiments, the water was replaced with 5 mM ion solutions, by mixing 275 μl of ions with 10 μl of vesicles and 15 μl of RsaA protein for a final ion concentration of 4.5 mM.

RsaA crystallization experiments with Langmuir-Blodgett air-water interface films. Recrystallization of RsaA at an air-water and lipid interface was attempted with a Fromherz-type Langmuir-Blodgett trough. The geometry of the apparatus has been previously described (9); each of eight compartments has a volume of 15 ml. The lipid monolayer was prepared in a manner similar to that previously described (18), except that the lipid-SLPS mixture in absolute ethanol (described above) was used. One drop of the lipid solution was spread on the air-water interface and compressed to a surface pressure of 25 to 30 mN m^{-1}. One milligram of RsaA protein, prepared by the low-pH extraction method was injected into the aqueous subphase of one compartment. The air-water interface, was sampled by application of carbon-stabilized, Pioloform-coated 400-mesh copper grids. Excess liquid was wicked away with filter paper, and the grids were negatively stained with 2% ammonium molybdate.

RESULTS AND DISCUSSION

Recrystallization of RsaA. Numerous attempts were made to improve the quality of in vitro recrystallization beyond that demonstrated previously (30), by using only purified RsaA protein (primarily prepared by the low-pH extraction method) and calcium ions. This process included variation in the amount of calcium ions added, as well as in protein concentration, incubation time, and temperature. Attempts to recrystallize on poly-L-lysine or bacitracin-treated grids were also carried out by pretreating Formvar-coated, carbon-stabilized grids with each reagent at 1 mg/ml followed by several rinses with water; recrystallization was then attempted by floating the grids on droplets containing mixtures of RsaA protein and calcium ions. None of the approaches was successful; the typical result showed mostly precipitated protein with only occasional patches or regions of low-resolution order. This result led us to theorize that a surface comparable to the natural bacterial surface was needed to at least initiate an efficient crystallization process.

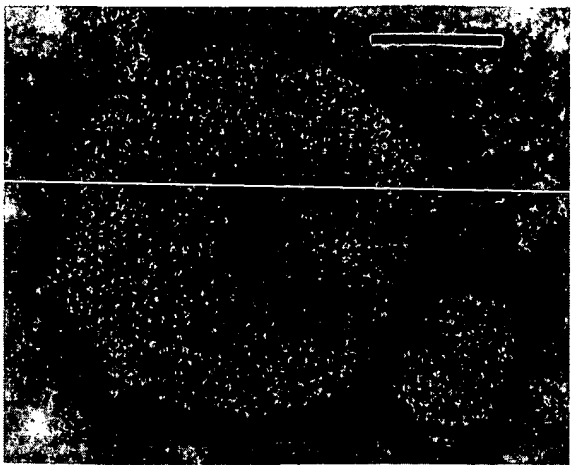

FIG. 1. Recrystallization of *C. crescentus* S-layer onto phosphotidylcholine-cholesterol-hexdecylamine-SLPS vesicles. Calcium ions (0.5 mM) and low-pH-extracted RsaA protein were used as described in Materials and Methods. Note that crystallization appears to start at several points and does not resolve when crystallization fronts meet. Bar, 0.2 μm.

When phospholipid-cholesterol vesicles were added to the mixture of RsaA and various concentrations of calcium ions, no change in quality of crystallization was detected, either on the surface of the vesicles or elsewhere in the preparation. But when the *Caulobacter* SLPS was added in the vesicle preparation stage, a dramatic change was effected. Frequently vesicles were completely covered with crystallized S-layer that showed good order, at least over short distances (Fig. 1). That is, it was common to see vesicles with a patchwork of S-layer crystallinity where it appeared that crystallization had initiated at numerous points and stopped as the patches joined one another.

The type of phospholipids did not seem to be important. The phosphatidylcholine used here is commonly used for preparation of artificial membranes, but phosphatidylglycerol and phosphoglucolipids are the predominant phospholipids in *C. crescentus* membranes (1, 21). Lipids from the archaebacterium *M. hungatei*, rich in a variety of tetraether lipids (29), were chosen as being as different as possible from the more typical phospholipids. Because the *M. hungatei* lipids were not soluble in absolute ethanol and SLPS was not soluble in chloroform, a slight modification of the vesicle preparation protocol was needed, but otherwise the results were comparable; RsaA crystallization was found only when SLPS was incorporated into the vesicles (Fig. 2). Indeed, we found that extensive good order was perhaps even more readily found on the surface of vesicles by using the *M. hungatei* lipids. We presume then that the exact ratio of phospholipid, cholesterol, and hexadecylamine was not a critical factor for successful S-layer crystallization.

Exploring the possibility that the SLPS promotion of RsaA recrystallization was not specific to this particular LPS, we also prepared phosphatidylcholine-type vesicles containing the rough (core sugars only) LPS of *C. crescentus* as well as a rough LPS and a complete (smooth) LPS from *Salmonella typhimurium*. No stimulation of recrystallization was noted in any of these cases.

If no calcium or other ions were added to the assay, only occasional and small patches of S-layer were noted. We attribute this to S-layer that was not completely dissociated during extraction and solublization (see below). As part of the demonstration that divalent ions, calcium in particular, were essential for the recrystallization, EGTA was added to the crystallization assay in one experiment to a final concentration of 2 mM. In this case, no S-layer was noted at all. This result is consistent with previous findings that EGTA disrupts S-layer crystals at concentrations in excess of 600 μM (28).

Preparation of RsaA used for in vitro recrystallization. Most experiments involving recrystallization of RsaA utilized protein prepared by the low-pH extraction method. This relatively mild procedure yielded RsaA that was about 90% pure, and remarkably, for a protein that spontaneously crystallizes, the protein would remain as a stable solution for 4 or more months. The method described above is a variation of that described before and provides more-detailed procedures because it became apparent as experimentation proceeded that the exact method of preparation had an influence on the stability and crystallization properties of the protein. The timing of pH neutralization after low-pH treatment was an important parameter; if one waited too long after low-pH treatment to neutralize (15 to 20 min or more), the protein spontaneously precipitated, and pH neutralization at that point did not arrest the precipitation. If the volume of low-pH solution was too small relative to the amount of cells treated, the result of using such a preparation for recrystallization was that small S-layer patches would form on nearly any type of vesicle surface, so long as divalent ions were supplied, but the protein solution was unstable and after 1 to 2 days the RsaA precipitated. We theorize that there likely was not enough protons to displace the calcium ions in the S-layer, and so the S-layer was not completely reduced to monomeric protein. Small, partly intact S-layer patches might then serve as effective nucleating points for the reassembly process, and so a surface for attachment was not needed. For cell cultures with cell densities resulting in optical absorbances of 1.5 to 2 at OD_{600}, resuspension of cell pellets in the low-pH solution at 20% of the original culture volume prevented this problem.

Eventually, however, the RsaA solutions accumulated more and more precipitated RsaA; when examined by negative stain microscopy, this protein appeared as poorly organized S-layer "crystalloids" and could be removed by centrifugation before use in the in vitro recrystallization assay. Since in all these cases the absolute number (but no more) of calcium or other divalent ions needed to accomplish recrystallization still remained in the solution, we theorize that gradually the ions found the

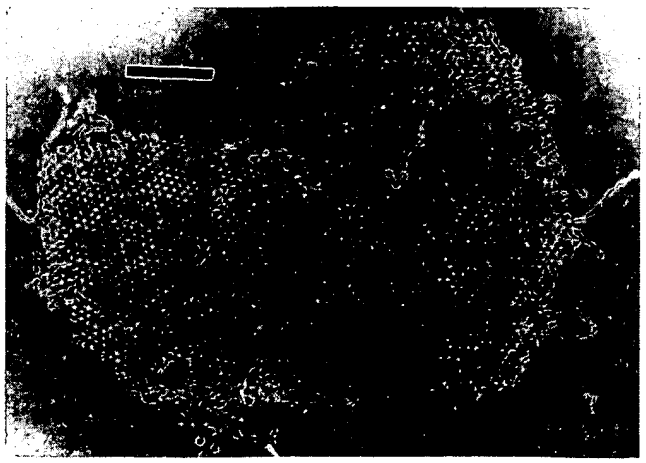

FIG. 2. S-layer recrystallization on *M. hungatei* lipid-SLPS vesicles. The vesicles routinely had a more flattened appearance on the electron microscope grid, compared to phosphotidylcholine vesicles, presumably a feature of the unusual lipids. Bar, 0.2 μm.

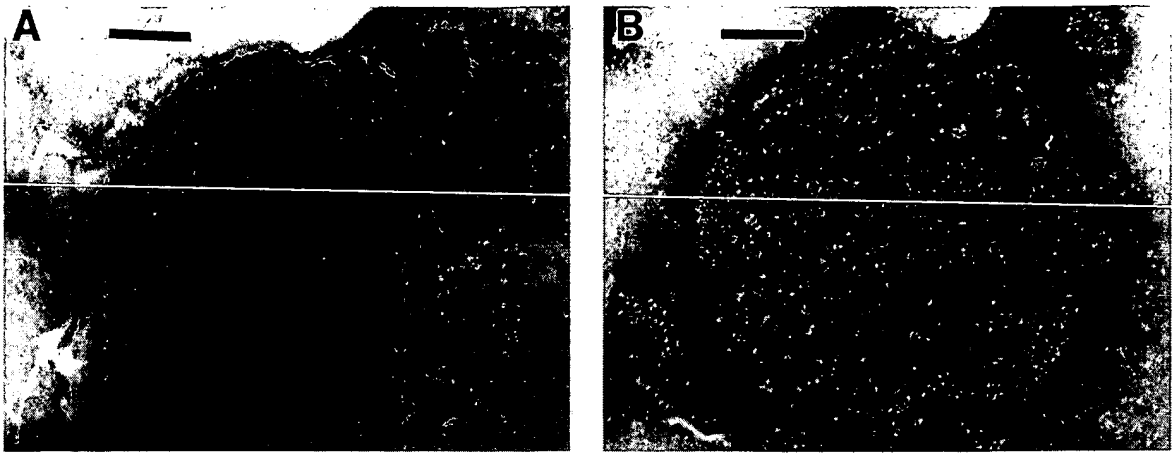

FIG. 3. S-layer recrystallization on phosphotidylcholine-cholesterol-hexdecylamine-SLPS vesicles, with cobalt (A) or manganese (B) ions. Bars, 0.2 μm.

proper position in a growing crystalloid. The addition of calcium to the protein solution greatly accelerated this process. On the other hand, as demonstrated in our previous work (30), large amounts of calcium (i.e., 10 mM) results in precipitated RsaA protein with a poor degree of crystallization, perhaps because the coalescence of calcium and protein occurs too rapidly.

RsaA protein that has been shed from cells forms a loose precipitate, composed of fibrils of RsaA protein, in liquid culture medium. This process was apparently an aberrant attempt to crystallize and occurs in particular in the S-layer shedding mutants, which lack the SLPS required for S-layer attachment (31). This precipitate was readily collected in nearly pure form but was refractory to the low-pH solublization. We therefore explored other methods of solublization. Treatment with 6 M guanidine-HCl was effective in solublizing shed RsaA, but upon dialysis, the protein readily precipitated. The use of 2% SDS led to solublization of the RsaA and a stable solution after dialysis to remove SDS, but the protein was completely inactive in a recrystallization assay.

Treatment with 8 M urea was more suitable; it was able to solubilize most or all precipitated RsaA, and when the protein concentration was adjusted to 1 to 2 mg/ml, the RsaA remained soluble after removal of the urea by dialysis. When soluble urea-derived RsaA protein was examined in the in vitro assay, it was found to crystallize onto vesicles in a manner analogous to that for the low-pH-derived protein, albeit at lower efficiency. That is, less crystallized protein was seen per unit of added protein and a corresponding increase in poorly crystallized or precipitated protein was noted. We have also been able to solubilize RsaA produced as inclusions in *E. coli*, expressed from a plasmid-borne *rsaA* gene copy, using the 8M urea method, and to achieve detectable recrystallization (28a).

Effect of alternative ions for S-layer recrystallization. We have learned from other studies that calcium likely has a role in the proper assembly of the S-layer (28, 31). In addition, it appears that availability of calcium is necessary for production of RsaA and is essential for the survival of caulobacters. The cellular target for this latter requirement is not known, but other ions can play the role that calcium plays; although with the exception of strontium, the alternate ions do not restore normal growth rate or membrane stability. For control of RsaA production, only strontium can replace calcium, and higher concentrations are required. These factors limited our ability to examine in vivo the range of ions that can substitute for calcium. The in vitro assay described here allowed a direct examination of the effect of ions on S-layer recrystallization.

The effect of various ions can be grouped into four classes. As expected, calcium and strontium ions resulted in the highest degree of recrystallization and thus formed a distinct class. This means that many or most vesicles exhibited extensive, well-ordered S-layers that produced good contrast by negative stain. This latter aspect seemed to reflect the fact that the S-layer was completely crystallized, with no missing subunits or gaps in the structure. The second class contains those ions that clearly stimulated S-layer formation, but the resulting S-layer was not as well ordered or contained missing segments or was not as extensively found in a preparation. Cobalt (Fig. 3), barium, manganese, and magnesium ions were grouped into this category. The third class contains those ions that produced a weak but perceptible stimulation of S-layer formation, clearly distinguishable from a control with no ions. These included nickel and cadmium. The fourth class included those ions that did not promote recrystallization and included the monovalent ions lithium, sodium, and potassium, in addition to zinc, aluminum, copper, and iron ions. These latter ions caused a precipitation of RsaA protein that was unrelated to normal crystallization and, we presume, may be inhibitory to recrystallization, even in the presence of a crystallization-promoting ion.

Several of the ions were also examined with 4.5 mM ion strength, instead of 0.5 mM. In no case was there an improvement in the quality or quantity of recrystallization.

In many ways the ability of various ions to substitute for calcium in recrystallization mirrors the success in substitution of calcium ions by other ions to permit growth. As detailed in a separate report (32), there is an unidentified physiological process that requires calcium, without which caulobacters do not grow. A variety of divalent and trivalent ions, when supplied at relatively high concentrations, can substitute, although both growth rates and apparent membrane stability are impaired. In that case monovalent ions were not able to substitute for calcium, and strontium was the best alternate ion.

S-layer recrystallization at an air-water interface. RsaA prepared by the low-pH extraction method was used to recrystallize the S-layer in a Langmuir-Blodgett apparatus. When SLPS was included with the phospholipid-cholesterol mix, recrystallization occurred readily, and via random sampling of the film that resulted, it appeared that with only 1 mg of protein, S-layer crystallization was nearly confluent over distances of

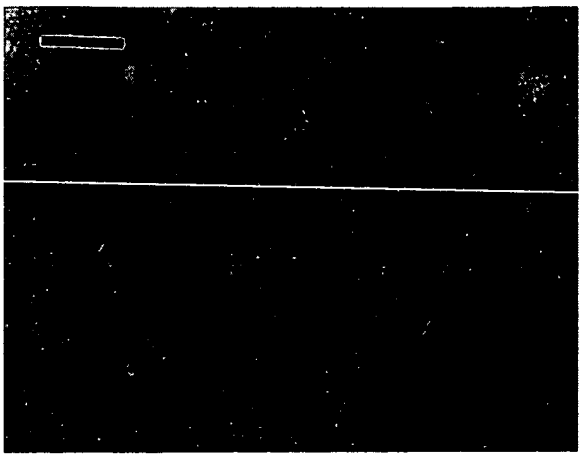

FIG. 4. S-layer recrystallization at the air-water interface in a Langmuir-Blodgett apparatus, with a phosphotidylcholine-cholesterol-hexdecylamine-SLPS lipid mixture. Although the uneveness of negative staining and the likely rearrangement of phospholipids during drying on the electron microscope grid are apparent, the degree of long-range recrystallization of RsaA is also apparent. Such images were typically found over nearly the entire surface of an electron microscope grid.

several square centimeters (Fig. 4). In contrast to the vesicle studies, there was no indication of a patchwork of recrystallization, with crystallized regions meeting one another. Instead, good order over long distances was the typical result. As with the studies using vesicles, the lipid mix without added SLPS resulted in no S-layer (data not shown). We presume in this case that the S-layer formed on the aqueous side of the lipid monolayer produced in the Langmuir-Blodgett apparatus such that the hydrophilic portion of the SLPS could form contacts with the forming S-layer, approximating the results from the vesicle-based recrystallization (see also reference 19).

General discussion. It was suggested in an earlier report that calcium may be involved with surface attachment, bridging the connection of crystallized RsaA with the SLPS in the outer membrane (31). But, it appears that is not the case; we have been unable to detect calcium binding to SLPS (data not shown) and so assume that calcium's role is in the subunit-subunit crystallization process, possibly mediated by the calcium binding motifs predicted from DNA sequence analysis (11).

The finding that SLPS is a key component for in vitro recrystallization of RsaA reenforces our previous assertion that SLPS is required for normal *Caulobacter* S-layer attachment (31). That assertion was based on the observation that mutant strains missing the SLPS were unable to attach the S-layer, but when colonies were examined by microscopy, crystallized S-layer arrays were found adjacent to cells. Such S-layer patches were mirror double layers; apparently in the conditions found within a colony, a stable two-dimensional array can form by using another similarly crystallizing layer as a substitute for SLPS anchored in the outer membrane. We do not yet know the basis for this double-layer interaction; it is apparently not based on calcium or SLPS binding to bridge the layers. A perhaps comparable situation occurs with the in vitro reassembly of the square S-layer lattice of *Bacillus sphaericus* CCM2177, where the two layers face each other bound by their net negatively charged inner faces (18).

Relatively little experimentation was done to vary the amount of SLPS in proportion to the phospholipid and cholesterol used to prepare vesicles. Because of the large difference in molecular size (the SLPS has a molecular mass of about 10,000 Da [28b]), exceeding a 1:200 SLPS-to-phospholipid mole ratio created problems in the incorporation of SLPS into the vesicles. With such a ratio it would seem impossible that SLPS was anchoring every possible binding site in the S-layer in these experiments. Nevertheless, it was common to see vesicles completely covered with closely adherent S-layer. This finding suggests to us that the SLPS, in concert with the remainder of the vesicle surface, serves as an anchoring point for the initialization of crystallization, which then continues without the requirement for numerous SLPS/S-layer attachments, so long as certain divalent ions (preferably calcium) and a surface are present. In support of this view was the patchwork of S-layer crystallinity described above, suggesting that recrystallization initiated from a limited number of points and terminated when fronts of advancing crystallization met. This type of phenomenon has also been noted in recrystallization experiments with isolated *Bacillus coagulans* S-layer proteins at air-water interfaces (17, 18).

This recrystallization assay will undoubtedly prove useful in future localized mutagenesis studies directed at identifying particular regions of the S-layer protein sequence responsible for surface attachment or monomer interactions that lead to crystallization. Yet it is recognized that interpretation must be approached with caution; although the end result of crystallized S-layer on a membrane surface may be similar or identical to the S-layer on a living cell surface, the processes that occur in the two instances are different. The in vitro assay involved recrystallization of protein that had been partly or largely denatured; the vesicles presented a static surface, and a lengthy time for assembly was permitted. This process contrasts with the native process in which newly made protein appears on the surface at a rapid rate, may still be in the process of folding for the first time, and assembles on the surface of a growing membrane. Indeed we have already experienced the result whereby a modified RsaA protein was able to crystallize in this assay but did not produce surface-attached S-layer on the cells producing the protein (4). Even so, the in vitro assay will be useful as a first test since it is much easier to assess S-layer crystallization by negative stain microscopy of vesicles than of whole cells and will be especially useful in situations where *C. crescentus* is unable to secrete modified RsaA proteins.

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